Int. J. Cancer (Pred. Oncol.): **74,** 31–34 (1997)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

SEROLOGICAL EVIDENCE OF AN ASSOCIATION BETWEEN *CHLAMYDIA PNEUMONIAE* INFECTION AND LUNG CANCER

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Epidemiological evidence suggests that airway obstruction is an independent risk factor for lung cancer and that this cannot be explained by active or passive smoking alone. *Chlamydia pneumoniae* infection has been associated with chronic bronchitis and its exacerbates. Our aim was to evaluate the association between chronic *C. pneumoniae* infection and risk of lung cancer among male smokers. Smoking males with lung cancer (n = 230) and their age- and locality-matched controls were selected among participants of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. The presence of *C. pneumoniae* infection was assessed by analyzing specific antibodies and immune complexes in 2 serum samples collected with a 3-year interval before the lung cancer diagnosis. The diagnosis of chronic infection was based on stable levels of positive specific IgA antibody (titer ≥ 16) and immune complex (titer ≥ 4). Relative risks were estimated by odds ratios (OR) adjusted for age, locality and smoking history by a conditional logistic regression model. Markers suggesting chronic *C. pneumoniae* infection were present in 52% of cases and 45% of controls and hence were positively associated with the incidence of lung cancer (OR 1.6; 95% confidence interval [CI] 1.0–2.3). The incidence was especially increased in men younger than 60 years (OR 2.9; 95% CI 1.5–5.4) but not in the older age group (OR 0.9; 95% CI 0.5–1.6). Before concluding that *C. pneumoniae* infection is a new independent risk factor for lung cancer, corroboration from other studies with larger number of cases and longer follow-up is needed. *Int. J. Cancer 74:31–34.*
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Chlamydia pneumoniae is a common intracellular bacterium that causes pneumonia and other respiratory infections world-wide. Like all *Chlamydia* organisms, it has a tendency to cause persistent and chronic infections. *C. pneumoniae* has been serologically associated with chronic lung diseases such as chronic bronchitis (von Hertzen *et al.,* 1995) and adult onset asthma (Hahn *et al.,* 1991), as well as and with atherosclerosis (Saikku *et al.,* 1988), in which the organism has been demonstrated in atherosclerotic plaques (Kuo *et al.,* 1993). Chronic bronchitis has been suggested as an independent etiologic factor in lung cancer (Osann, 1991), the effect of which cannot be explained by either active or passive exposure to cigarette smoke. Smoking does, however, appear to promote chronic *C. pneumoniae* infection, probably through immunosuppression (Karvonen *et al.,* 1994*a*).

In Finland, nearly 30,000 male smokers were followed for 5–8 years in 1985–1993 in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC Study Group, 1994). We analyzed baseline and 3-year follow-up serum samples from 230 men diagnosed with lung cancer after the third year and their age-, time- and locality-matched controls for *C. pneumoniae*-specific IgG and IgA antibodies and immune complexes to study the association between markers suggestive of chronic *C. pneumoniae* infection and lung cancer.

MATERIAL AND METHODS

Base population and follow-up

The ATBC study was a randomized, double-blind, placebo-controlled prevention trial that examined whether supplementation with alpha-tocopherol, beta-carotene or both would reduce the incidence of lung cancer in male smokers. A cohort of 29,133 men 50–69 years of age who were current smokers (5 or more cigarettes/day at entry) was randomly divided into 4 supplementation regimens: alpha-tocopherol (50 mg/day) alone, beta-carotene (20 mg/day) alone, both alpha-tocopherol and beta-carotene or placebo. The cohort was recruited from the total male population of this age group in South-Western Finland (N = 290,406) from 1985 through 1988. Potential participants with a history of cancer or serious disease limiting their ability to participate were excluded. The presence of chronic obstructive pulmonary disease was not, however, an exclusion criterion. Those taking supplements of vitamin E, vitamin A or beta-carotene in excess of predefined doses and those being treated with anticoagulant agents were also excluded. Before their enrollment, the participants were interviewed at one of 14 local study centers to obtain details of their medical, smoking, dietary and occupational histories and were scheduled for a chest X-ray intended to identify and exclude existing lung cancers. Serum samples were collected for each participant at baseline and 3 years after the randomization and stored at −70°C.

Eight hundred and ninety cohort members developed lung cancer during the follow-up of 5–8 years (median 6.1 years) through April 30, 1993. Cases were identified through the Finnish Cancer Registry, and diagnostic information for each lung cancer was reviewed by the Clinical Review Committee for confirmation and staging. Tissue histology was reviewed by 2 study pathologists and cytology by 2 study pulmonary cytologists. Histology was available for 76% of the lung cancer cases, cytology for 18% and clinical data alone for 6%.

Selection of cases and controls

There were 426 men in whom lung cancer was diagnosed after the second blood sampling at 3 years. For each of these, one control subject was selected from those participants of the cohort who were at risk at the time of the lung cancer diagnosis of the case and had both the baseline and the 3-year blood sample taken and stored. The control subject was further matched for age (±1 year), supplementation group, study center and timing of the baseline and follow-up blood sampling (±100 days). Thus 393 eligible case-control pairs were obtained. Due to practical constraints, it was not possible to include all of them. Hence, a random sample of 249 pairs was drawn, and 230 pairs had sufficient serum sample for analysis.

Serological studies

C. pneumoniae-specific serum IgG and IgA antibodies were determined by the micro-immunofluorescence method using *C.*

Contract grant sponsor: National Cancer Institute, contract grant number NO1-GN-45165; Contract grant sponsor: Academy of Finland.

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Received 24 June 1996; Revised 4 October 1996

pneumoniae strain Kajaani 6 and *Chlamydia trachomatis* strain L2, elementary bodies as antigens and fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (Kallestad, Chaska, MO) and IgA (Sigma, St. Louis, MO) as antibodies. The serum samples were analyzed at 4-fold dilutions starting at 1:32 for IgG and 1:16 for IgA. Immune complexes (IC) were isolated by polyethylene glycol 6000 (PEG; Fluka, Buchs, Switzerland) precipitation (Linnanmäki *et al.*, 1993). In brief, 100 µl of the sample were added to an equal volume of 7% PEG in sodium borate buffer, pH 8.4, and the mixture was incubated overnight at 4°C followed by centrifugation at 4,500*g* for 15 min. The pellets were then washed twice with 3.5% PEG-borate. Finally, the precipitates were dissolved to the original volume in phosphate-buffered saline (PBS). The immune complexes thus obtained were then analyzed by microimmunofluorescence for the presence of *C. pneumoniae* antibodies at 2-fold dilutions starting at 1:2. The antibody and immune complex determinations of each case and the individual control were always tested simultaneously in the same titration series in a blinded fashion.

Chronic *C. pneumoniae* infection was defined as persistent IgA antibodies and immune complexes, based on earlier experience (Saikku *et al.*, 1992; Linnanmäki *et al.*, 1993). An IgA titer of ≥ 16 and an IC titer of ≥ 4 present in both baseline and 3-year samples was considered strong evidence for chronic *C. pneumoniae* infection. Furthermore, when the IgA titer was ≥ 16 in both samples irrespective of the IC titer, or when the IC titer was ≥ 4 in both samples and the IgA titer ≥ 16 only in the latter sample, the findings were accepted as moderate evidence of chronic infection. IgG titers were measured to show the overall prevalence of *C. pneumoniae* antibodies in this population.

Statistical analyses

The analysis of lung cancer risk in relation to the evidence of chronic *C. pneumoniae* infection was based on keeping the original 230 matched pairs as the units of analysis. A conditional logistic regression model was used in estimating the odds ratios (OR) associated with evidence of infection, taking into account the matching factors (age, supplementation group, study center and timing of serum sampling) and adjusting for self-reported years of smoking and daily consumption of cigarettes. Taking no evidence as the reference category, the ORs were estimated both separately for strong and moderate evidence of chronic *C. pneumoniae* infection and for strong or moderate evidence combined. The possible monotonic trend in the ORs over the levels of infection evidence was tested by treating the latter as a quantitative variable, assuming scores 0, 1 and 2 for none, moderate and strong evidence, respectively, and contrasting the residual deviance statistic of that model to the model excluding infection evidence. Possible modification of the relative risk by age, histologic type (smoking-related squamous cell and small cell carcinomas *vs.* the other types), time interval between the first serum sample and cancer diagnosis (< 5 years *vs.* ≥ 5 years) and smoking was analyzed by including appropriate interaction terms in the models. The reported OR estimates for the association between evidence for *C. pneumoniae* infection and lung cancer specific to age, histology and time interval from the first sample until the date of cancer diagnosis were computed from separate analyses in the respective subgroups. The models were fitted using the GLIM 3.77 statistical program.

RESULTS

At the baseline, the 230 lung cancer cases and their matched controls were very similar in their distributions of age (mean 60.3 years in both), years of regular smoking (mean 40.7 *vs.* 38.1 years) and daily cigarette consumption (mean 21.5 *vs.* 19.7), although the number of cigarettes per day and years of smoking were slightly higher among the cases (Table I). Of the cases, 99 (46%) were squamous cell carcinomas, 55 (25%) small cell carcinomas, 34 (16%) adenocarcinomas and 28 (13%) other carcinomas; 14 cases had no histological or cytological diagnosis.

TABLE I – DISTRIBUTION (%) OF CASES AND CONTROLS BY AGE, YEARS OF REGULAR SMOKING AND DAILY CONSUMPTION OF CIGARETTES AT BASELINE

Parameter	Cases (n = 230)	Controls (n = 230)
Age		
50–54	17	19
55–59	28	26
60–64	36	36
65–69	19	19
Years of smoking		
<25	2	6
25–34	11	21
35–44	53	48
≥45	34	25
Cigarettes per day		
5–14	14	27
15–24	49	41
25–34	30	24
≥35	7	8

TABLE II – DISTRIBUTION (%) OF *C. pneumoniae*-SPECIFIC ANTIBODY AND IMMUNE COMPLEX (IC) TITERS AND THE GEOMETRIC MEAN TITERS (GMT) OF CASES AND CONTROLS AND THE PREVALENCE OF COMBINED IgA AND IC POSITIVITY IN BASELINE AND 3-YEAR SERUM SAMPLES

Antibody titer	Cases		Controls	
	Baseline (n = 230)	3-year (n = 230)	Baseline (n = 230)	3-year (n = 230)
IgG				
<32	2	2	5	5
32–128	56	62	56	58
>128	42	37	39	37
GMT	180	160	157	147
IgA				
<16	41	44	49	54
16–64	37	38	35	31
>64	22	18	16	15
GMT	32	30	24	22
IC				
<2	11	8	15	12
2–<4	19	16	17	19
≥4	70	76	68	69
GMT	5.1	5.4	4.9	4.9
IgA ≥16 and IC ≥4	44	47	37	34

Table II shows the distribution and geometric mean titers of *C. pneumoniae* antibodies and immune complexes (IC) in the baseline and 3-year serum samples of the cases and controls. The IgG titers were positive (≥ 32) in 98% of cases and in 95% of controls in both baseline and 3-year samples. Also, in pairwise comparisons, the IgG, IgA and IC titers of the cases were somewhat higher than those of the controls; 37% of the cancer cases and 31% of the controls had strong evidence for chronic *C. pneumoniae* (IgA ≥ 16 and IC ≥ 4 in both samples), and 16% and 14%, respectively, had moderate evidence (either IgA ≥ 16 in both samples irrespective of IC, or IC ≥ 4 in both samples with IgA ≥ 16 only in the latter sample). Antibodies against *C. trachomatis* were less common, with IgG titers for *C. trachomatis* being positive (≥ 32) in only 11% of the cases and in 13% of the controls in the first sample and in 9% and 10%, respectively, in the second sample.

The distribution of the matched pairs with discordant evidence for infection status indicated that the lung cancer risk was overall positively associated with chronic *C. pneumoniae* infection, more so among subjects aged < 60 years at baseline (Table III). The estimated OR, contrasting subjects in the combined category of strong or moderate evidence with those who had no evidence, was 1.6 (95% CI 1.0–2.3) for all lung cancers, adjusted for the matching factors, daily number of cigarettes and years of smoking. When comparing different histological types, a higher relative risk was observed in the combined group of small cell and squamous cell carcinomas (OR 1.7; 95% CI 1.0–2.8) than in the other cancer types

(OR 1.3; 95% CI 0.7–2.7), but the observed heterogeneity of the OR estimates by histologic type was within random error. Stratification by age indicated a particularly high relative risk for all lung cancer types in cases younger than 60 years at recruitment (OR 2.9; 95% CI 1.5–5.4), whereas no indication of an association between infection with lung cancer among the older men was observed (OR 0.9; 95% CI 0.5–1.6; $p < 0.01$ for the interaction of age and infection evidence). The relative odds for those cases cancer found at least 5 years after the first serum sample (OR 1.4; 95% CI 0.4–2.8) was somewhat lower than that for cases of cancer diagnosed after a shorter period (OR 1.7; 95% CI 1.0–2.8), but these estimates were also consistent with an assumption of similar relative risk by length of follow-up. There was no evidence of modification of the relative risk associated with the infection status by daily number of cigarettes and years of smoking. When the 3-point scale for evidence of infection was used, the results indicated a monotonic trend in risk with respect to the strength of infection evidence for cancer, primarily among the young men (< 60 years; Table IV). An increasing trend was observed in the 2 broad histological categories, particularly in small cell and squamous cell carcinomas, but for other cancer types the evidence for trend was somewhat weaker. In cancers diagnosed within 5 years since the first serum samples an increasing trend was also found but not in cancers occurring after a longer period. The confidence intervals were, however, wide, so the results were also consistent with an assumption of no modification of trend by this time interval.

DISCUSSION

Cigarette smoking is the most important etiologic factor of lung cancer. Chronic bronchitis and other previous lung diseases are known risk factors for lung cancer, in both smokers and non-smokers (Osann, 1991). In the present study, markers of chronic *C. pneumoniae* infection, both stable elevated IgA and immune complexes, were found to be associated with lung cancer among regular smokers. Adjustment for daily consumption and years of smoking did not alter the results. These findings suggest that chronic *C. pneumoniae* infection is an independent risk factor for lung cancer.

The internal validity of our study is in many respects rather good. The base population was a well-defined geographical cohort. All key measurements were performed within a narrow time interval with virtually complete coverage. The follow-up for cancer incidence through the nation-wide cancer registry was also complete. Differential misclassification regarding both exposure and outcome assessment was eliminated by the uniform, appropriately blinded procedures. Some degree of non-differential misclassification probably existed in the exposure assessment, the likely effect of it being to dilute the association toward null. Confounding due to cigarette smoking was controlled both by restriction of the study to smokers only and by adjusting for the effects of daily number of cigarettes and years of smoking in the logistic regression model. Matching controlled for age, place of residence and circumstances relevant to the exposure measurement. Some residual confounding due to possible associations between other exposures to carcinogenic substances (*e.g.*, radon) and *C. pneumoniae* infection may still remain; however, its role is likely to be negligible. On the other hand, the statistical precision of our relative risk estimates was not as high as one would hope, due to the small numbers of discordant pairs.

The increased risk associated with *C. pneumoniae* infection appeared to be limited to younger men (< 60 years), but the confidence intervals about the OR estimates in older men were concordant with a positive association in this age group, too. This attenuation of relative risk by age is also observed between *C. pneumoniae* infection and chronic coronary disease (Saikku *et al.*, 1988, 1992), and it would be consistent with the possibility of an additive rather than a multiplicative combination of the effects of *C. pneumoniae* and age.

Before any strong causal interpretations of the observed association between the evidence of *C. pneumoniae* infection and lung cancer, one must also take in account that the time between serum sampling and clinical manifestation of the cancer was relatively short (*i.e.*, 3–8 years for baseline sample and less than 5 years for the follow-up sample). Within this time range we could not find any significant difference in the relative risk between a longer and a shorter follow-up. Given this short time, it is conceivable that the higher prevalence of infection among the cancer patients was in fact caused by an immunosuppressive effect of the latent cancer itself. However, the differences in the relative risk estimates in different age groups are not entirely in line with this explanation. A longer follow-up after serum samples would be needed to obtain more information on this issue.

Analogous evidence exists in support of chronic bronchitis being an independent risk factor for lung cancer (Osann, 1991). Chronic inflammation associated with various persistent infections has long been considered a risk factor in the development of malignancy. For example, the part played by viruses is well recognized and has been intensively studied (zur Hausen, 1991), and a common bacterium causing chronic inflammation of the gastric wall, *Helicobacter pylori*, has been associated with gastric carcinoma (Correa *et al.*, 1990). For some time chronic chlamydial infections of other types have been suspected to be associated with cancers. Beginning in the 1930s, the lymphogranuloma venereum biovar of *C. trachomatis*, causing chronic rectal inflammation, was associated with rectal cancer (Levin *et al.*, 1964). Genital *C. trachomatis* infections have also been associated with cervical cancer (Jha *et al.*, 1993), an association shown to be independent of papillomavirus infection (Hakama *et al.*, 1993). In this study, we have no information concerning persistent viral infections, and it is as-

TABLE III – FREQUENCY DISTRIBUTION OF THE MATCHED 230 PAIRS ACCORDING TO THE EVIDENCE FOR CHRONIC *C. pneumoniae* INFECTION IN BOTH CASES AND CONTROLS[1]

Infection evidence in lung cancer cases	Infection evidence in control subjects			
	Strong	Moderate	No	Total
Strong	28 (10)	12 (6)	49 (29)	89 (45)
Moderate	10 (4)	7 (3)	20 (12)	37 (19)
No	30 (7)	15 (7)	59 (25)	104 (39)
Total	68 (21)	34 (16)	128 (66)	230 (103)

[1]Numbers in parenthesis refer to the pairs aged <60 years at baseline.

TABLE IV – NUMBERS OF MATCHED PAIRS OF LUNG CANCER CASES AND CONTROL SUBJECTS AND THE ADJUSTED ODDS RATIO (OR) ESTIMATES WITH THEIR 95% CONFIDENCE INTERVALS (CI), CONTRASTING THOSE WITH STRONG OR MODERATE EVIDENCE TO THOSE WITH NO EVIDENCE OF CHRONIC *C. pneumoniae* INFECTION, ACCORDING TO HISTOLOGICAL TYPE OF THE LUNG CANCER, AGE OF THE CASE AND LENGTH OF THE FOLLOW-UP SINCE FIRST SERUM SAMPLE UNTIL THE LUNG CANCER DIAGNOSIS

	Total number of pairs	OR (95% CI)[1]		*p* value for trend[2]
		Moderate *vs.* no evidence	Strong *vs.* no evidence	
All cancers	230	1.4 (0.8–2.5)	1.6 (1.1–2.6)	0.03
Small cell and squamous cell carcinomas	154	1.5 (0.7–2.9)	1.8 (1.0–3.2)	0.03
Other cancers	76	1.3 (0.4–4.1)	1.4 (0.7–2.8)	0.4
Age <60 years	103	1.9 (0.8–4.4)	3.8 (1.8–8.3)	0.001
Age ≥60 years	127	1.0 (0.4–2.4)	0.9 (0.5–1.6)	0.62
Follow-up <5 years	138	1.2 (0.6–2.5)	2.0 (1.1–3.6)	0.01
Follow-up ≥5 years	92	2.2 (0.7–6.7)	1.2 (0.5–2.5)	0.54

[1]Adjusted for the matching factors (age, study center, treatment group and timing of samples), daily number of cigarettes and years of regular smoking in conditional logistic regression models.–[2]Calculated from a model with quantitative scores (0, 1, 2) for the levels of infection evidence.

sumed that the association of *C. pneumoniae* infection and lung cancer we observed is independent of other infections.

The persistence of elevated antibody titers is generally considered to be a sign of chronic infection (Buck *et al.,* 1986; Brett *et al.,* 1990; Saikku *et al.,* 1992). After an acute *C. pneumoniae* infection, IgG antibody titers rise and usually decrease slowly, whereas IgA antibodies tend to disappear rapidly, the half-life of IgA being only 5–6 days. In reinfection, IgA response is often prominent. Elevated IgA titers have been considered a reliable marker of chronic bacterial infection of *Pseudomonas aeruginosa* in cystic fibrosis (Brett *et al.,* 1990), *H. pylori* in gastritis (Buck *et al.,* 1986) and *C. pneumoniae* in chronic bronchitis (von Hertzen *et al.,* 1995) and coronary heart disease (Saikku *et al.,* 1992). Furthermore circulating immune complexes containing microbial components are often observed in chronic infections (Höby *et al.,* 1986). On this basis, elevated IgA and immune complex titers were also considered markers of chronic *C. pneumoniae* infection in the present study.

In Scandinavia *C. pneumoniae* causes slow widespread epidemics every 5–7 years. During interepidemic periods, 5–10% of all pneumonia cases in adults are caused by *C. pneumoniae,* while during the peak months of an epidemic chlamydia can be associated with up to 45% of community-acquired pneumonias. In this study, the baseline serum samples were collected during an epidemic period (Karvonen *et al.,* 1994*b*), and this was reflected in higher antibody titers in the first sample compared with those taken 3 years later in both lung cancer cases and controls.

Viruses probably induce malignancy by their oncogenic effects on cells (zur Hausen, 1991). The connection of bacterial infections to the development of malignancy is less clear. Genetic damage and neoplastic transformation are shown to be induced *in vitro* by co-culturing cells with activated inflammatory cells (Rosin *et al.,* 1994). Oshima and Bartsch (1994) have suggested that nitric oxide and other free radicals released by activated inflammatory cells have a role in the carcinogenesis induced by *H. pylori* infection in the stomach. The same mechanism has been suggested for the role of schistosomiasis in the development of urinary bladder carcinoma (Rosin *et al.,* 1994). The liberation of nitric oxide has also been shown to occur in chlamydial infections (Mayer *et al.,* 1993). The possibility that chronic *C. pneumoniae* infection induces carcinogenesis in the lung through mediators of inflammation could thus be a logical consequence from a chronic infection.

Our study population was restricted to regular smokers. Hence we cannot evaluate whether the effects of *C. pneumoniae* were different in non-smokers, so at this stage we have no adequate basis to generalize these results to non-smokers.

In conclusion, markers suggesting chronic *C. pneumoniae* infection were associated with lung cancer in smokers. This association was more evident for cases with small cell and squamous cell carcinoma and in men younger than 60 years. The findings suggest that chronic *C. pneumoniae* infection may be a new independent risk factor for lung cancer, but corroboration from other studies with larger numbers of cases and longer follow-up periods after *C. pneumoniae* antibody measurements are needed. Whether it is an important risk factor in non-smokers, too, remains to be investigated as well.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dr. P.H. Mäkelä for her invaluable criticism on the manuscript. The study was supported by grants from the National Cancer Institute (contract NO1-CN-45165) and the Academy of Finland.

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